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Prolactin, Growth Hormone, and Epidermal Growth Factor Activate Stat5 in Different Compartments of Mammary Tissue and Exert Different and Overlapping Developmental Effects

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Prolactin (Prl)-induced phosphorylation of Stat (signal transducer and activator of transcription) 5 is considered a key event in functional mammary development and differentiation. We now demonstrate that not only Prl, but also growth hormone (GH) and epidermal growth factor (EGF), can activate Stat5 in mammary tissue. We investigated the roles of these hormones in mammary development using mice in which the respective receptors had been inactivated. Although Prl receptor (PrlR)-null mice are infertile, we were able to maintain pregnancies in a few mice by treatment with progesterone. Mammary tissue in these mice was severely underdeveloped and exhibited limited differentiation as assessed by the phosphorylation status of Stat5 and the expression of milk protein genes. PrlR +/− mice showed impaired mammary development and alveolar differentiation during pregnancy, which corresponded with reduced phosphorylation levels of Stat5a and 5b, and impaired expression of milk protein genes. Development of the glands in these mice was arrested at around day 13 of pregnancy. While Prl activated Stat5 only in the epithelium, GH and EGF activated Stat5 preferentially in the stroma. To assess the relevance of the GH receptor (GHR) in the mammary gland, we transplanted GHR-null epithelium into cleared fat pads of wild-type mice. These experiments demonstrated that the GHR in the epithelium is not required for functional mammary development. Similarly, the EGFR in the epithelium is not required for alveolar development. In contrast, epithelial PrlR is required for mammary development and milk protein gene expression during pregnancy. Although GH is not required for alveolar development, we were able to demonstrate its lactogenic function in cultured mammary epithelium from PrlR-null mice. However, ductal development in GHR-null mice was impaired, supporting the notion that GH signals through the stromal compartment. Our findings demonstrate that GH, Prl, and EGF activate Stat5 in separate compartments, which in turn reflects their specific roles in ductal and alveolar development and differentiation.

INTRODUCTION

Development of the mammary gland is controlled by systemic steroid and peptide hormones and local growth modulators (Topper and Freeman, 1980). Early physiological findings on the requirement of specific hormones, such as estrogen, progesterone, prolactin (Prl), and oxytocin, have been supported by more recent gene deletion studies in the mouse (for reviews see Hennighausen and Robinson, 1998; Robinson *et al.*, 2000a). We have previously demonstrated that the presence of the transcription factor Stat (signal transducer and activator of transcription) 5a is required for the functional development of mammary tissue (Liu *et al.*, 1997). Both alveolar proliferation and functional differentiation are impaired in the absence of Stat5a, and the underlying defect is autonomous to the epithelium (Liu *et al.*, 1998). In addition to Prl, epidermal growth factor (EGF) and growth hormone (GH) can also activate Stat5a in mammary

cells *in vitro* (Tourkine *et al.*, 1995; David *et al.*, 1996). As a general concept, cytokines and growth factors bind to their respective receptors and elicit their activation by either autophosphorylation (EGF receptor) or Jak2-mediated phosphorylation (GH and Prl receptors). Stat5a and 5b in turn are recruited to these membrane receptors and phosphorylated by activated Jak2 (GH and Prl signaling) (Ihle, 1996) or by Src kinases (EGF receptor) (Olayioye *et al.*, 1999). Once activated, Stat5 proteins form homo- and heterodimers that translocate to the nucleus where they mediate the transcription of specific genes. Although the genes that are activated by these cytokines are largely unknown it is believed that they initiate programs of cell proliferation, differentiation, and survival.

Genetic verification that prolactin controls mammary gland development and function was provided by gene deletion experiments in the mouse (Horseman *et al.*, 1997; Ormandy *et al.*, 1997). Deletion of both copies of the Prl gene renders the female infertile and thus prohibits studies during pregnancy and lactation. Similarly, mice from which both copies of the PrlR had been deleted are infertile (Ormandy *et al.*, 1997). Transplantation of PrlR-null epithelium into wild-type mice demonstrated a cell-autonomous defect of alveolar proliferation, during pregnancy (Brisken *et al.*, 1999). Mice carrying one copy of the inactive PrlR are fertile but fail to develop a functional mammary gland during their first pregnancy (Ormandy *et al.*, 1997; Brisken *et al.*, 1999), suggesting that a threshold level of the receptor is required for normal development.

The EGF receptor (EGFR) family consists of four members, which are recognized by a large group of ligands, the EGF-related peptides (Salomon *et al.*, 1995; Riese and Stern, 1998). Genetic evidence that the EGFR contributes to mammary development came from the waved-2 mice, which carry a point mutation in the gene (Fowler *et al.*, 1995). The genetic inactivation of a combination of several other ligands confirmed and extended this study to show unique and redundant functions (Luetteke *et al.*, 1999). Since EGFR-null mice die perinatally (Threadgill *et al.*, 1995), mammary transplant experiments were performed to identify the role of this receptor. Using combinatorial transplants into renal capsules of host mice it was demonstrated that ductal morphogenesis and branching, but not lobuloalveolar development of the mammary gland, was dependent upon the presence of the EGFR in the mammary stroma. However, mammary epithelium devoid of the EGFR developed normally in wild-type gland-free fat pads (Wiesen *et al.*, 1999).

GH can activate Stat5 through its receptor in liver (Gronowski *et al.*, 1996; Choi and Waxman, 1999) and in primary mammary epithelial cells (Tourkine *et al.*, 1995). Although a role for GH in mammary development and in lactogenesis had been suspected (Plaut *et al.*, 1993; Flint and Gardner, 1994; Zebrowska *et al.*, 1997; Flint and Vernon, 1998; Kann *et al.*, 1999) genetic evidence had been lacking. The availability of GHR-null mice (Zhou *et al.*, 1997) now permitted the evaluation of the role of GH on mammary development.

In an effort to understand the physiology of Prl, GH, and EGF in mammary tissue, we investigated whether these cytokines could activate the Jak2/Stat5 pathway in mammary epithelium and stroma *in vivo*. In addition, we investigated the role of the respective receptors in mammary tissue through the transplantation of GHR-, PrlR-, and EGFR-null epithelium into control stroma, followed by biochemical and molecular experiments on Stat5 activation and milk protein gene expression, respectively. Finally we investigated the nature of the developmental and lactational defects in PrlR-hemizygous females.

MATERIALS AND METHODS

Animals. EGFR hemizygous mice (CD1 background) (Threadgill *et al.*, 1995) were kindly provided by Dr. Stuart H. Yuspa (NCI, NIH, Bethesda, MD) and the other models were prepared in our laboratories (Ormandy *et al.*, 1997; Zhou *et al.*, 1997). All mice used in the cytokine injection experiments, as donors and recipients in PrlR and GH transplantations, and in explant culture experiments were in the C57BL/6 background. We used CD1 outbred and NCI athymic nu/nu mice as donors and recipients, respectively, in the EGFR fetal mammary gland transplants. The PrlR-null mice were in the 129/SvJ or C57/BL6 background, and the GHR-null mice were originally in the 129Ola/BalbC background and were bred into C57/BL6 background. Mice were housed in a 12-h day/night cycle at 22°C and 80% relative humidity with food and water *ad libitum*. For all pregnancy experiments, day 0 of pregnancy was considered the day on which the vaginal plug was found.

Determination of genotypes. The determination of genotypes was done by PCR. The three primers used for GHR analysis were In3+1 primer, 5′-CCT CCC AGA GAG ACT GGC TT-3′; In4−1 primer, 5′-CCC TGA GAC CTC CTC AGT TC-3′; Neo-3 primer, 5′-GCT CGA CAT TGG GTG GAA ACA T-3′. Genomic DNA was amplified under the following conditions: initial 5 min at 95°C; 30 cycles of 95°C for 30 s, 58°C for 45 s, 72°C for 60 s; and a final extension for 5 min at 72°C. The primers used for EGFR analysis were Egfr-25, 5′-GCC CTG CCT TTC CCA CCA TA-3′; Egfr-26, 5′-TTG CAG CAC ATC CCC CTT TC-3′; Egfr-27, 5′-ATC AAC TTT GGG AGC CAC AC-3′. Genomic DNA was amplified under the following conditions: initial 2 min at 95°C; 35 cycles of 96°C for 20 s, 65°C for 30 s; and a final extension for 7 min at 72°C. The primers used for PrlR analysis were AS, 5′-GAA GAG CAA GAT CTC AAG AAC-3′; Neo, 5′-CCA GTC CCT TCC CGC TTC AGT-3′; 5F, 5′-GAG AAA AAC ACC TAT GAA TGT C-3′. Genomic DNA was amplified under the following conditions: initial 5 min at 94°C; 35 cycles of 95°C for 45 s, 55°C for 60 s, 72°C for 45 s; and a final extension for 5 min at 72°C.

Cytokine injections. Rat GH, rat Prl, and mouse recombinant Prl were injected at a concentration of 5 μg/g of body weight (provided by Dr. A. Parlow, Director of the National Hormone and Pituitary Program, Harbor–UCLA Medical Center, Torrance, CA). Human recombinant EGF was injected at a concentration of 10 μg/g of body weight (kindly provided by Dr. Jorcano, CIEMAT, Spain).

Progesterone implants. Progesterone with biodegradable carrier-binder (Innovative Research of America) was administered

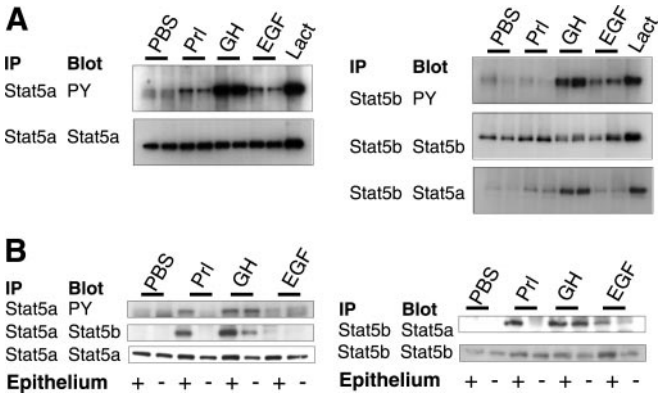

FIG. 1. Analysis of Stat5a and 5b phosphorylation and formation of heterodimers in mammary tissue 15 min after injection of mature female mice with PBS, prolactin (Prl), growth hormone (GH), or epidermal growth factor (EGF). (A) Immunoprecipitations of mammary protein extracts with anti-Stat5a antibodies (left) and anti-Stat5b antibodies (right) followed by Western blot probing with anti-phosphotyrosine, anti-Stat5a, or anti-Stat5b antibodies. The duplicated lanes correspond to samples from two different mice injected with the respective cytokine, as indicated. (B) Immunoprecipitations of cellular proteins with anti-Stat5a (left) or Stat5b (right) antibodies. Proteins from whole mammary tissue (epithelium +) or epithelium-free mammary stroma (epithelium −) were extracted from right and left fourth mammary gland from the same animal after the respective cytokine injection, as indicated. Protein extracts were immunoprecipitated and Western blots were performed with anti-phosphotyrosine, anti-Stat5a, or anti-Stat5b antibodies, as indicated.

to 129/SvJ, PrlR-null females, in 5-mg pellets that permitted a 3-week release (Binart *et al.,* 2000).

Organ cultures of mouse mammary tissue. Four-month-old virgin PrlR-null and control mice were sacrificed and their glands were excised. Mammary explants were cultured for 48 h in DMEM supplemented with the indicated hormones as described previously (Yoshimura and Oka, 1990). The concentrations of hormones used were as follows: insulin, 5 μg/ml; hydrocortisone, 1 μg/ml; ovine Prl (NIH hormone service), 5 μg/ml; and rGH, 5 μg/ml.

RT-PCR. Total cellular RNA was purified from 30 to 75 mg of cultured mammary gland explant using TRIzol Reagent (GIBCO BRL, Life-Technologies, Inc., Rockville, MD). Total RNA (0.5 μg) was reverse-transcribed with Thermoscript (GIBCO BRL, Life-Technologies, Inc.) with oligo(dT) primers at 55°C for 45 min. Synthesized cDNA was diluted to 42 μl, and 2 μl was used for PCR. β-Casein-specific primers for RT-PCR were CasYP (ACT ACA TTT ACT GTA TCC TCT GAC), nucleotides 107 to130 of mouse β-casein coding sequence, and CasYM (GTG CTA CTT GCT GCA GAA AGT ACA G), nucleotides 620 to 644. cDNA was amplified under the following conditions: initial 2 min at 94°C; 28 or 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 60 s; and a final extension for 5 min at 72°C.

As a positive internal control, the same amount of cDNA was amplified for 22 cycles using primers specific to GAPDH cDNA, RGAPH-1 (GTG AAG GTC GGT GTG AAC GGA TTT GGC CGT), nucleotides 50 to 76 of mouse GAPDH sequence, and RGAPH-2 (CCA CCA CCC TGT TGC TGT AG), nucleotides 997 to 1016. As a negative internal control, the same amount (23.8 ng) of RNA without reverse transcription was amplified for 28 or 35 cycles using CasYP and CasYM. Water without template cDNA was also amplified as a negative control. Obtained PCR fragments were run on a 2% agarose gel and stained with ethidium bromide.

Transplantation of mammary epithelium. Transplants from GHR- and PrlR-null mammary epithelium were performed as previously described (DeOme *et al.,* 1959). Fetal mammary transplants from EGFR KO and control 14-day-old fetuses were performed as described (Robinson *et al.,* 2000b).

Histology. Mammary tissue was harvested from cervically dislocated mice. For whole-mount examination, the tissue was fixed in Carnoy's solution for 4 h and stained with carmine alum overnight as described previously (Kordon *et al.,* 1995). Whole mounts were embedded and sectioned using standard methods. For histological analysis mammary sections were stained with H&E.

Gene expression. Protein and RNA analyses, protein extraction from mammary tissues, immunoprecipitations, and Western blotting were performed as described previously (Liu *et al.,* 1996). Antibodies for Stat5a, Stat5b, and phosphotyrosine have been described (Liu *et al.,* 1996). Total RNA was isolated and Northern blots were performed as described previously (Robinson *et al.,* 1995).

RESULTS

GH, EGF, and Prl Activate Stat5a in Mammary Tissue

To determine the ability of GH and EGF to activate Stat5 in mammary tissue *in vivo,* we injected 7-week-old virgin mice with recombinant human EGF, purified rat GH, or

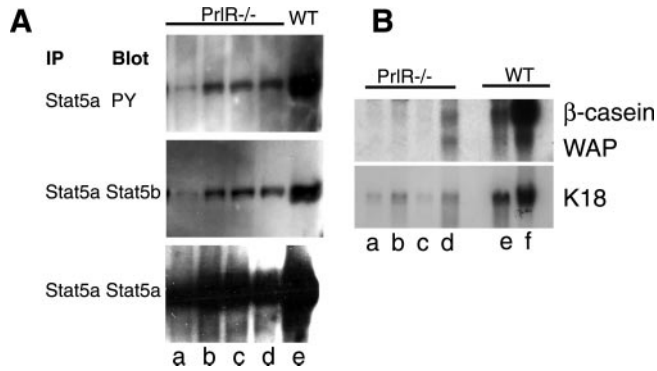

FIG. 2. Stat5a activation and milk protein gene expression in PrlR-null mice. (A) Cellular proteins from mammary tissue from PrlR-null pregnant mice were precipitated with anti-Stat5a antibodies, followed by Western blot analysis with anti-phosphotyrosine (top), anti-Stat5b (middle), and anti-Stat5a (bottom) antibodies. Tissue was harvested from PrlR-null mice at days 13 (a), 15 (b), 17 (c), and 18 (d) of pregnancy and from pregnancy day 18 (e) of a wild-type mouse. (B) Northern blot analysis of β-casein and WAP mRNA in mammary tissue of PrlR-null mice. Lanes a–d correspond to lanes a–d in A. Mammary tissue from a day 13 (e) and a day 18 (f) pregnant wild-type mouse was analyzed. The membrane was reprobed with keratin 18 (bottom), a marker of the epithelial component of the gland.

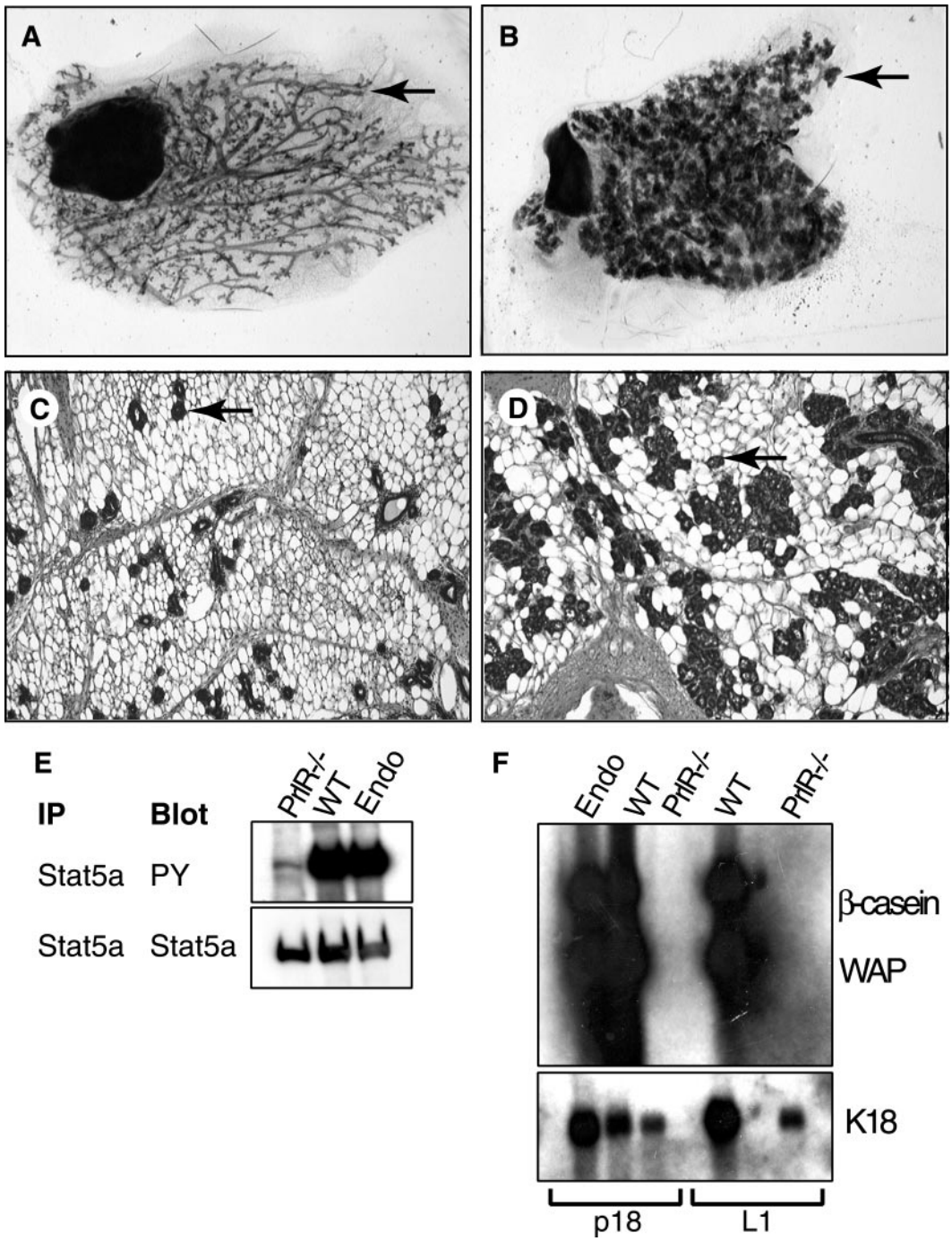

FIG. 3. Mammary development and function in PrlR-null epithelial transplants. Mice were mated 8 weeks after transplanting the epithelium and sacrificed at day 18 of pregnancy or immediately after parturition. (A–D) Whole-mount and histology of PrlR-null (A and C) and control (B and D) epithelial transplants at pregnancy day 18. PrlR-null (A) and wild-type (B) transplants from the same recipient were fixed with Carnoy's, stained with carmine alum, and photographed at 16×. Sections from the whole mounts were stained with H&E and photographed at 100× (C and D). Considerably fewer epithelial structures can be observed in PrlR-null epithelial transplants (C) than in controls (D). The arrows in A and B point to lobuloalveolar structures and in C and D to individual alveoli. (E and F) Stat5a activation and milk protein gene expression in PrlR-null epithelial transplants. (E) Immunoprecipitation of cellular proteins from PrlR-null and wild-type transplants and from an endogenous gland from the recipient mouse. Immunoprecipitation of cellular proteins was performed with anti-Stat5a antibody and Western blots were performed with anti-phosphotyrosine (top) or anti-Stat5a (bottom) antibodies. (F) Northern analysis of β-casein and WAP mRNA at day 18 of pregnancy and after parturition of PrlR-null and control epithelial transplants and endogenous host mammary tissue. The membrane was reprobed with keratin 18 (bottom) as a control of mammary epithelial content in the transplants. Endo, endogenous, third mammary gland. WT, wild-type epithelial transplant. PrlR −/−, PrlR-null epithelial transplant. K18, keratin 18. p18, pregnancy day 18. L1, lactation day 1.

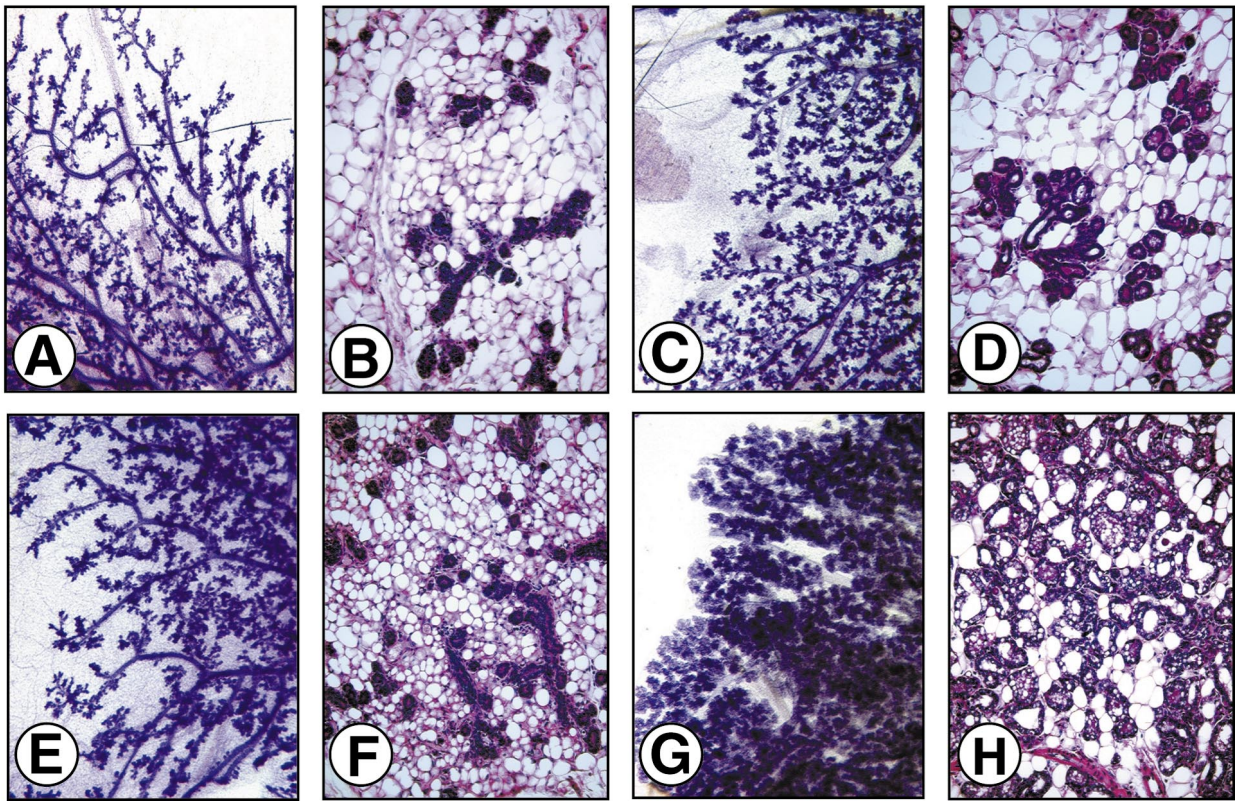

FIG. 4. Whole-mount and histological analysis of alveolar morphogenesis during the first pregnancy in PrlR +/− (A–D) and wild-type (E–H) mice. Glands were analyzed at day 12 of pregnancy (A, B, E, and F) and at day 1 after parturition (C, D, G, and H). (A, C, E, and G) Whole mounts were stained with carmine alum and photographed at 20×. (B, D, F, and H) Histological sections from the whole mounts were stained with H&E and photographed at magnification 200×.

purified rat Prl. After 15 min, mammary glands were isolated and we analyzed the phosphorylation status of Stat5a and 5b, the formation of 5a–5b heterodimers, and the respective steady-state protein levels (Fig. 1A). For each cytokine mammary tissue from two independent mice was analyzed as shown in juxtaposed lanes. While Prl activated Stat5a in virgin females, Stat5b phosphorylation was barely detectable (Fig. 1A). The presence of Stat5a–5b heterodimers confirmed the existence of activated Stat5b after Prl injection (Fig. 1A). The highest level of Stat5a and Stat5b phosphorylation, however, was detected after GH injection (Fig. 1A). EGF induced phosphorylation of both Stat5a and Stat5b (Fig. 1A). Heterodimers between Stat5b and 5a were observed mainly after GH treatment and to a lesser extent after EGF and Prl treatment (Fig. 1A). The protein levels of Stat5a and Stat5b did not change significantly upon hormone injection (Fig. 1A). GH and EGF induction of Stat5a and Stat5b activity was validated in liver tissue from the same animals, since EGF and GH phosphorylation of Stat5 in liver has been described (Ruff-Jamison *et al.,* 1995; Gronowski *et al.,* 1996). EGF and GH administration elicited equally high levels of Stat5a and Stat5b phosphorylation, demonstrating that both cytokines were injected at a dose sufficient to produce high Stat5a and Stat5b phosphorylation levels (data not shown). In contrast, Prl induced a fast depletion of the basal levels of Stat5 in liver (data not shown).

Although EGF, Prl, and GH activate Stat5a and Stat5b in mammary tissue, it is not clear whether the epithelium, the stromal fat pad, or both compartments respond to the cytokine signals. To distinguish between these possibilities we removed mammary epithelium from one inguinal (No. 4) gland of a 3-week-old mouse and left the contralateral gland intact. Recombinant EGF, rat GH, or recombinant mouse Prl was injected into these mice at the age of 7 weeks, and Stat5a phosphorylation was assessed 15 min after the injection. Prl activated Stat5a and Stat5b, as measured by tyrosine phosphorylation and heterodimerization, in the intact gland, but failed to do so in the cleared fat pad (Fig. 1B). GH activated Stat5a and 5b at similar levels in the intact gland and in the cleared fat pad (Fig. 1B). Low levels of Stat5a activity were detected after EGF injection (Fig. 1B). Comparing the Stat5a phosphorylation status with the ability of Stat5a to form heterodimers with Stat5b

revealed differences between the whole gland and the stromal compartment upon GH injection. Stat5a efficiently formed heterodimers with Stat5b after GH injection, in the intact gland, and, to a lesser extent, in the cleared fat pad (Fig. 1B). This indicates that GH–Stat5 signaling is also active in the epithelial compartment. In contrast, equal amounts of Stat5b formed heterodimers with Stat5a in the intact gland and the cleared fat pad (Fig. 1B). This may reflect that all Stat5b present in the gland forms heterodimers. Thus, Stat5b appears to be the limiting molecule for the formation of heterodimers.

Role of the Prolactin Receptor in Mammary Gland Development

Since PrlR-null mice are infertile, Ormandy and his colleagues evaluated development of PrlR-null mammary epithelium by transplanting it into wild-type hosts (Brisken *et al.*, 1999). They demonstrated that reduced epithelial development occurred in the absence of the receptor in the epithelial compartment. Nevertheless, mammary development in the physiological context of PrlR-null mice remains to be investigated. This is important, because a functional overlap may exist between distinct, but interrelated pathways (Liu *et al.*, 1998). We have now studied Stat5 activation and milk protein gene expression in PrlR-null mice in which pregnancy had been restored (Binart *et al.*, 2000). In addition, we have performed transplant experiments and evaluated epithelial development on a functional level by measuring milk protein expression.

Stat5a activation and milk protein gene expression in PrlR-null mice. As Prl is involved in progesterone metabolism, the disruption of PrlR signaling produces a deficiency in progesterone levels that prevents maintenance of pregnancies. Subcutaneous delivery of progesterone enabled the maintenance of pregnancies in a few mice. Although progesterone had a beneficial effect on the maintenance of embryos during the first half of pregnancy, an increasing number of resorption sites were seen between days 12 and 19. Despite the fact that a large number of embryos were lost from midgestation, Stat5a phosphorylation was detected in mammary tissue from PrlR-null mice as early as day 13 of pregnancy (Fig. 2A, lanes a–d). Nevertheless, at day 18 of pregnancy, phosphorylation levels were much lower than in control mice (Fig. 2A, lane e). Stat5a also formed heterodimers with Stat5b in the PrlR-null mice (Fig. 2A, lanes a–d). WAP and β-casein mRNA were detected in mammary tissue of a PrlR-null mouse at day 18 of pregnancy (Fig. 2B, lane d) although at a much lower level than in comparable control mice (Fig. 2B, lane f).

Stat5a activation and milk protein expression in transplanted PrlR-null epithelium. Mammary transplant experiments (Brisken *et al.*, 1999) had shown that PrlR-null epithelium forms normal ducts in wild-type hosts, but fails to develop lobuloalveolar structures during pregnancy. However, the status of Stat5 phosphorylation and expression of milk protein genes had not been explored. We transplanted PrlR-null epithelium into cleared fat pads of 20 nulliparous mice and examined the mammary glands at day 18 of pregnancy (P18) and day 1 after parturition (L1), assessing morphological development, phosphorylation of Stat5, and steady-state levels of β-casein and WAP transcripts. Whole-mount analyses of the transplants at P18 showed normal ductal elongation and branching but sparse and empty alveoli compared to contralateral control transplants (Figs. 3A and 3B). The histology of the transplants showed one-fourth as many alveoli and poor overall differentiation of the alveoli (Figs. 3C and 3D). To quantify the degree of functional differentiation of the PrlR-null alveoli, Stat5a phosphorylation was assayed. We detected only traces of phosphorylation compared to the strong signal obtained in the transplanted control gland and the endogenous gland of the host mouse (Fig. 3E). The presence of milk protein mRNAs constitutes another marker of functional mammary gland differentiation. RNA was extracted from transplanted mammary tissue at P18 and L1, followed by Northern blot analysis for WAP and β-casein mRNA. No expression of these RNAs was detected in PrlR-null tissue at either time point (Fig. 3F). As a control for epithelial content in the transplants we analyzed for keratin 18 mRNA expression. Keratin 18 is present in the ductal and alveolar cells.

Mammary development and function in PrlR +/− mice. Mice with only one copy of the PrlR (PrlR +/−) exhibit incomplete mammary development during pregnancy and fail to lactate (Ormandy *et al.*, 1997; Brisken *et al.*, 1999). At that point it was not known whether the lack of a full complement of PrlR results in delayed development or a block at a specific stage. We monitored mammary gland development in PrlR +/− and control mice from P12 to L1. Little difference in development was seen between control (Figs. 4E and 4F) and mutant (Figs. 4A and 4B) tissue at P12. Both PrlR +/− and control mice had an extended ductal tree with some lobuloalveolar decorations. At P14 and P18 extended lobuloalveolar structures were abundant in control mice but not in PrlR +/− mice (data not shown). At L1 the fat pad of control mice was filled with lobuloalveolar structures (Figs. 4G and 4H) but PrlR +/− alveoli had not progressed much further (Figs. 4C and 4D).

To determine the degree of differentiation we measured WAP and β-casein mRNA levels at P18 and L1. While both WAP and β-casein RNA were abundant in control tissue at P18 (Fig. 5A, lane a), expression in two independent PrlR (+/−) mice was reduced by approximately 90% (Fig. 5A, lanes c and d). WAP expression was not detectable in PrlR (+/−) mice at this time point. The presence of low levels of β-casein mRNA and the absence of WAP mRNA were similar to what was observed in wild-type mice at P14 (Burdon *et al.*, 1991). Although WAP and β-casein mRNA levels increased in hemizygous mice at parturition (Fig. 5A, lane e), they reached only 10% of the level seen in control mice. After parturition PrlR +/− mice expressed WAP and β-casein RNA at equivalent levels, whereas in control mice the levels of WAP RNA exceed those of β-casein. This

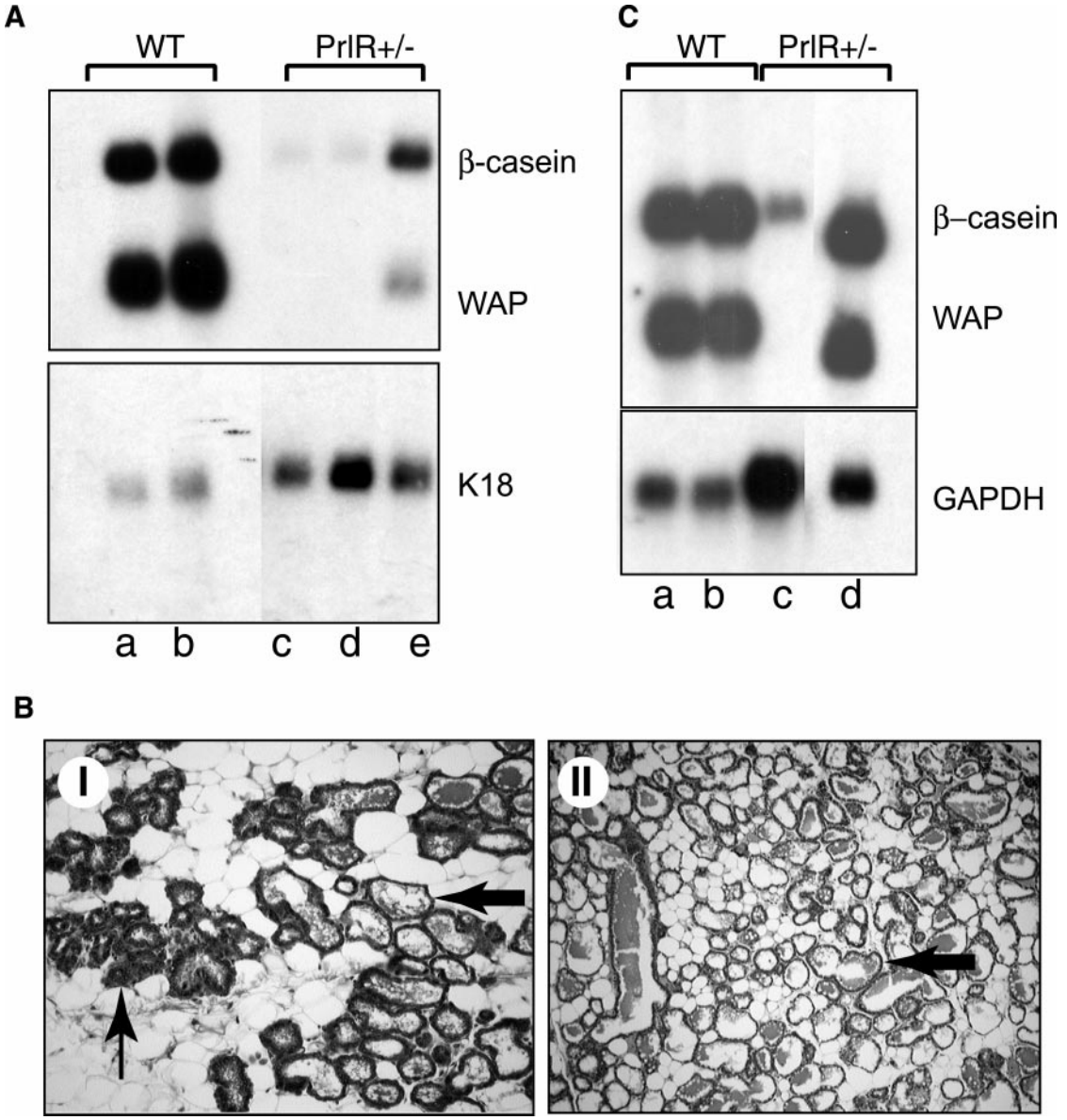

FIG. 5. Histology and milk protein gene expression in PrlR +/− mice after the 1st and 10th pregnancy. (A) Northern analysis of WAP and β-casein mRNA in PrlR +/− and wild-type mice during their first pregnancy. Lanes a and b, wild-type mice at day 18 of pregnancy and after parturition, respectively. Lanes c and d, samples from two PrlR +/− mice at pregnancy day 18. Lane e, PrlR +/− mouse at postpartum day 1. (B) Histology of a PrlR +/− mouse that had gone through 10 pregnancies (I) and wild-type mouse (II). (I) Alveoli are sparse and while some are expanded and full with milk (thick arrow) others are empty (thin arrow). (II) All alveoli are well developed (thick arrow). (C) Northern analysis of β-casein and WAP mRNA in a PrlR +/− mouse during its 1st (lane c) and 10th pregnancy (lane d). Lanes a and b, wild-type mouse at day 18 of first pregnancy.

further indicated that the mutant alveolar cells do not reach full differentiation.

PrlR +/− mice in a mixed 129/C57BL/6 background are able to lactate following the second pregnancy (Ormandy *et al.*, 1997; Brisken *et al.*, 1999), suggesting that continued hormonal stimulation could overcome developmental blocks. We investigated this possibility in the C57BL/6 background. None of the more than 30 PrlR +/− mice analyzed were able to lactate after their 1st or 2nd pregnancy, and from the 15 mice that had gone through multiple pregnancies only 1 mouse was able to lactate after 9 pregnancies. Whole-mount analysis of mice that had gone through more than 6 pregnancies revealed mammary tissue with a heterogeneous appearance. In some areas alveoli

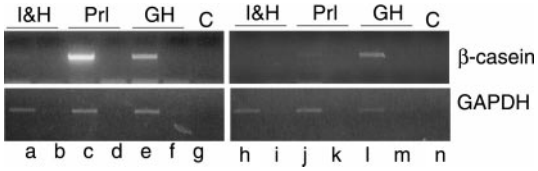

FIG. 6. RT-PCR analysis of β-casein and GAPDH mRNA in PrlR-null mammary gland explants after culture in the presence of insulin and hydrocortisone alone or with ovine Prl or rat GH. Mammary tissue from 4-month-old virgin control (a, b, c, d, e, f) and PrlR-null (h, i, j, k, l, m) mice was dissected and cultured for 48 h in culture medium supplemented with different hormones, as indicated. Lanes a, b, h, and i, insulin and hydrocortisone; lanes c, d, j, and k, insulin, hydrocortisone, and Prl; lanes e, f, l, and m, insulin, hydrocortisone, and GH. Total cellular RNA from PrlR-null and wild-type cultured explants was purified, and cDNA was synthesized and analyzed with β-casein-specific primers (top, lanes a, c, e, h, j, l) or with GAPDH-specific primers (bottom, lanes a, c, e, h, j, l). As a negative internal control RNA was amplified without the prior RT step using the β-casein primers (top, lanes b, d, f, i, k, m) or with GAPDH-specific primers (bottom, lanes b, d, f, i, k, m). Water without template cDNA was also amplified as a negative control (C, lanes g and n).

were extended as in control mice, while other areas exhibited poor development (data not shown). Histological analysis revealed areas densely populated with alveoli and other rather sparse regions (Fig. 5BI). Some lobules appeared to be fully developed and were expanded with milk, while others appeared immature with little secretion (Fig. 5BI). One PrlR +/− mouse in the C57BL/6 background was successful in nurturing her pups after 9 pregnancies. High levels of WAP and β-casein mRNA were detected at day 18 of the 10th pregnancy in the same mouse (Fig. 5C, lane d). While Stat5 phosphorylation in mammary tissue from late-pregnant PrlR +/− mice after their 1st pregnancy was low, normal phosphorylation levels were observed in the mouse that could partially lactate after its 9th pregnancy (data not shown).

GH can activate β-casein transcription in PrlR-null mice. Although GH is known to be a lactogenic hormone (Plaut *et al.*, 1993; Flint and Gardner, 1994; Zebrowska *et al.*, 1997; Flint and Vernon, 1998), it is not clear whether this signaling proceeds through its own receptor or through the PrlR. To distinguish between these possibilities we performed explant culture experiments with PrlR-null mammary epithelium in the presence and absence of GH. Mammary tissue from 4-month-old virgin PrlR-null and control mice was cultured for 48 h in the presence of insulin and hydrocortisone alone or with rat GH or ovine Prl. β-Casein mRNA was detected in control and PrlR-null explants that were cultured with rat GH (Fig. 6, lanes e and l). No expression was detected in PrlR-null explants cultured with Prl (Fig. 6, lane j) or in control and PrlR-null explants cultured with insulin and hydrocortisone alone (Fig. 6, lanes a and h). The presence of contaminant genomic DNA in the RNA samples was excluded by analyzing RNA in the absence of reverse transcriptase (Fig. 6, lanes b, d, f, i, k, and m). This result demonstrates that GH is able to activate milk protein expression independent of PrlR signaling in the mouse and therefore GH synergizes with Prl in the lactogenic process.

Role of GH in Mammary Gland Development

We investigated the role of GH in mammary gland development using GHR-null mice (Zhou *et al.*, 1997) and transplants of GHR-null epithelium into wild-type stroma. Ductal outgrowth in 11-week-old GHR-null mice was greatly retarded and the ducts had barely reached the lymph node, while the fat pad was filled in control littermates (Figs. 7A and 7B). At the age of 15 and 23 weeks the ducts had reached the limit of the fat pad, but they were thinner than their wild-type counterparts and side branching was limited (data not shown).

In order to investigate whether the defects observed in GHR-null mammary glands were due to the lack of GHR in the epithelial compartment we performed transplantation experiments of GHR-null mammary epithelium into cleared fat pads of syngenic mice. Wild-type (wt) epithelium was grafted into the cleared contralateral glands as an internal control. Puberty-mediated ductal outgrowth and branching of GHR-null and wild-type transplants was investigated 8 weeks after the transplantation. There was no significant difference between GHR-null and control glands at the virgin stage (Figs. 7C and 7D). In addition no differences were observed in lobuloalveolar proliferation and differentiation between GHR-null and wt transplants (data not shown). To validate these histological findings we determined Stat5a and Stat5b phosphorylation as a differentiation marker during late pregnancy and at parturition. High levels of Stat5 phosphorylation were detected in mammary tissue from GHR-null mice (Fig. 7E, lanes d, e, and k) as well as in GHR-null transplanted epithelial outgrowths (Fig. 7E, lanes g and m). These experiments demonstrate that epithelial GHR is not necessary for ductal branch morphogenesis or alveolar differentiation of the mammary gland.

Role of the EGFR in Mammary Gland Development

Mammary development cannot be studied easily in EGFR-null mice because they die between early embryonic stages and perinatally dependent on the genetic background (Threadgill *et al.*, 1995b). Cunha and colleagues used the mammary transplant approach with postnatal tissue to evaluate the contribution of the EGFR in stroma versus epithelium (Sebastian *et al.*, 1998; Wiesen *et al.*, 1999) and they identified a critical role for the EGFR within the stroma but not the epithelium. Since the severity of EGFR mutations is highly depen-

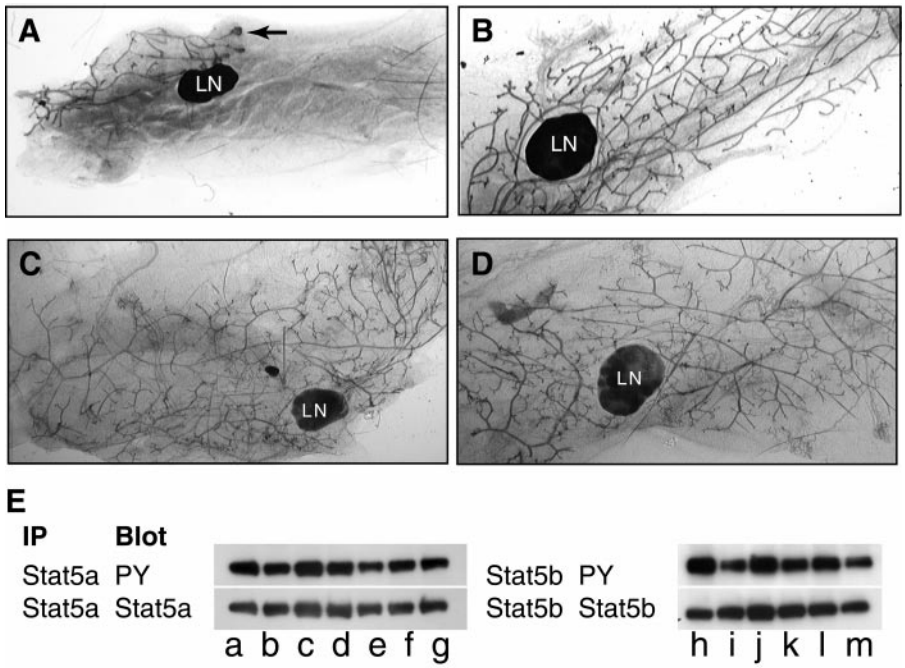

FIG. 7. Mammary development and function in GHR-null mice and GHR-null epithelial transplants. (A and B) Whole-mount analysis of ductal outgrowth and branching in GHR-null mammary glands (A) and corresponding wild-type mice (B). The arrow in A shows how far the epithelial tree has grown. (C and D) Whole-mount analysis of ductal outgrowth and branching morphogenesis in GH-null (C) and control (D) epithelial transplants. GHR-null and control epithelium were grafted into opposite glands of the same recipient and harvested 8 weeks after surgery. Magnification is 10×. LN, lymph node. (E) Biochemical analysis of alveolar development in GHR-null mouse mammary tissue, GHR-null epithelial transplants, and wild-type epithelial transplants during pregnancy and at parturition. Immunoprecipitation of cellular proteins with anti-Stat5a (left) and anti-Stat5b (right) antibodies. Western blots were performed with anti-phosphotyrosine (top) or anti-Stat5a and anti-Stat5b antibodies (bottom, as indicated). Lanes a and h, wild-type mice, day 1 of lactation. Lanes b and i, wild-type mice, day 17 of pregnancy. Lanes c and j, wild-type mice, day 18 of pregnancy. Lanes d and k, GHR-null mice at day 17 of pregnancy. Lane e, GHR-null mouse at day 20 of pregnancy (could not deliver). Lanes f and l, wild-type epithelial transplant at lactation day 1. Lanes g and m, GHR-null epithelial transplant at lactation day 1.

dent upon the strain background (Sibilia and Wagner, 1995) we performed mammary transplantation experiments in the outbred CD1 strain. EGFR-null mice in this strain die perinatally and we transplanted fetal mammary tissue to study ductal and alveolar development during puberty and pregnancy. EGFR-null fetuses at E18.5 display rudimentary ductal structures in the nipple area (Fig. 8A). In contrast, extensive ductal elongation and branching can be seen in control fetuses at E18.5 (Fig. 8B). Nevertheless when transplantation experiments of fetal epithelial tissue from EGFR-null and control mice were performed in the fat pads of wild-type nude mice, no differences were found in ductal development or alveolar differentiation between EGFR-null and wild-type epithelium (data not shown). This confirmed the observation by Wiesen and colleagues obtained in the 129SV/J × Swiss Black background that EGFR in mammary epithelium is not required for ductal and alveolar development.

DISCUSSION

Signaling through the prolactin receptor is critical for functional mammary gland development (Ormandy *et al.*, 1997). Mice lacking both copies of the PrlR are infertile and thus it has not been possible to investigate mammary development within this genetic context. Previous studies demonstrated a very low level of progesterone in PrlR-null mice serum, leading to a defect in the maintenance of pregnancy to completion. Upon systemic treatment with progesterone, a few PrlR-null mice maintained their pregnancies and we observed limited mammary development (Binart *et al.*, 2000). Histological and biochemical evaluation revealed limited alveolar development and differentiation in the mammary gland. However, we detected greatly reduced levels of phosphorylated Stat5a and 5b proteins and decreased levels of β-casein and WAP transcripts. Since these mice are devoid of any functional PrlR, other lacto-

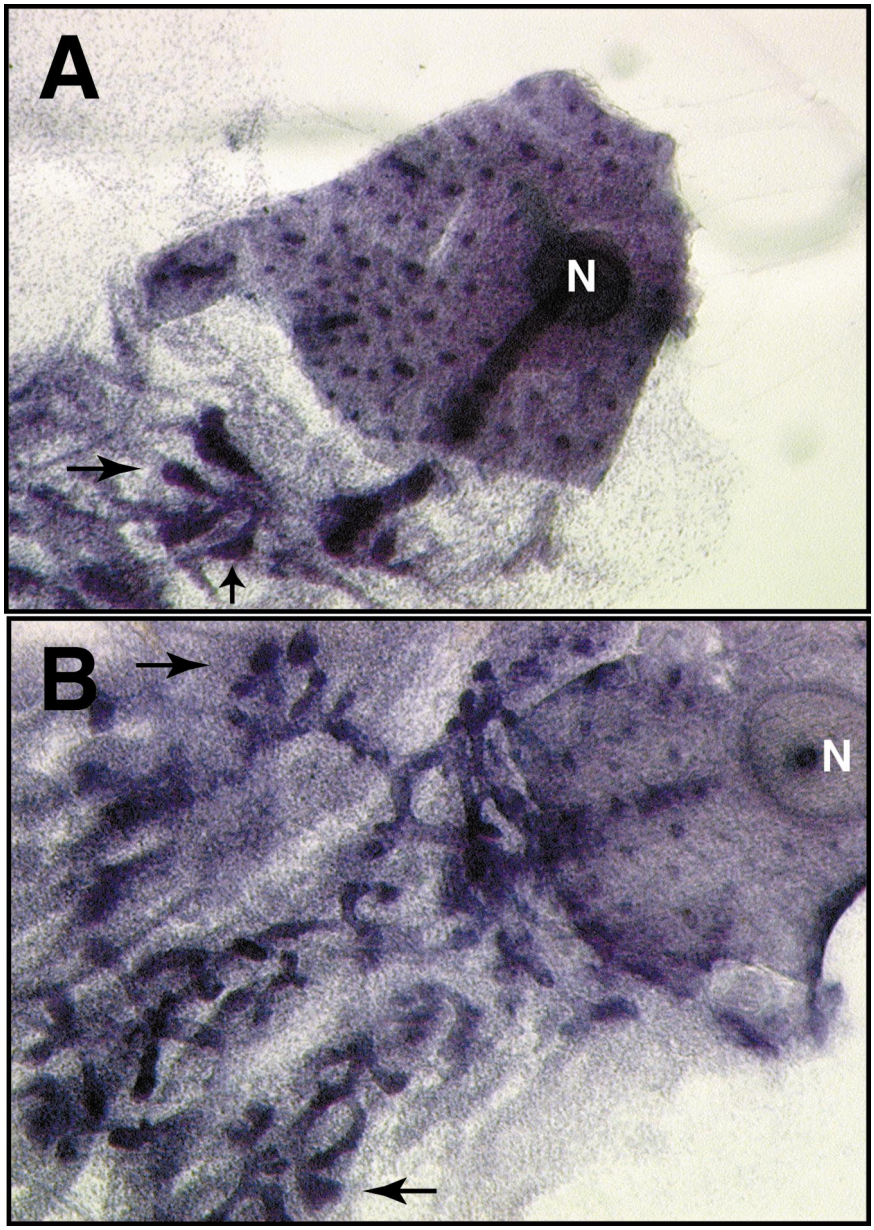

FIG. 8. Whole-mount analysis of EGFR-null (A) and wild-type (B) fetal mammary glands (E18). The glands were stained with carmine alum and photographed at 50×. N marks the location of the nipple and arrows point to end-bud structures. The dots in the skin are hair follicles.

genic stimuli must activate these signaling pathways within the epithelium. Both EGF and GH have been suggested to contribute to the developmental program of the mammary gland (Coleman *et al.*, 1988; Snedeker *et al.*, 1991; Plaut *et al.*, 1993; Schroeder and Lee, 1998). We now show that EGF and particularly GH can activate Stat5 in mammary tissue of virgin mice. While Prl induced Stat5 phosphorylation in the whole gland but not in the epithelium-free fat pad, EGF and GH activated Stat5 at comparable levels in the whole gland and cleared fat pad. This suggests that both GH and EGF convey developmental cues through the epithelium and the stroma. In contrast, Prl signaling is autonomous to the epithelium (Brisken *et al.*, 1999).

GH has been suspected to contribute to the development of the mammary gland. However, the nature of its action is not fully understood and it is unclear whether it signals through a local or a systemic mechanism (Feldman *et al.*, 1993, 1999; Walden *et al.*, 1998; Ruan and Kleinberg, 1999). Our analysis of virgin GHR-null mice demonstrates that

GH is necessary for ductal outgrowth and branching during mammary gland morphogenesis. This deficiency is overcome during pregnancy and GHR-null mice can feed their pups. Based on mammary transplantation experiments we demonstrate that GHR-null ductal epithelium can fully develop. The stunted growth in the GHR-null mice is therefore the consequence of aberrant systemic and/or stromal signals. Although the GHR is dispensable for functional development of mammary tissue during pregnancy and the maintenance of lactation, its presence could account for the limited development observed in the absence of the PrlR. GH can activate β-casein in mammary explant cultures from PrlR-null mice, suggesting that this pathway participated in the limited mammary development in the absence of any Prl signals. The lactogenic activity of GH may be elicited directly through the activation of Stat5 followed by the activation of milk protein gene transcription in alveolar cells or indirectly through insulin-like growth factor expression (Ruan *et al.*, 1992, 1995; Plaut *et al.*, 1993; Ruan and Kleinberg, 1999). Since GH can activate Stat5 in the epithelium and the stroma, we suggest that both mechanisms are operative.

Although the EGFR is considered a mediator of epithelial proliferation, mammary transplantation experiments have demonstrated that EGFR-null mammary epithelium undergoes normal growth and differentiation when grafted into wild-type stroma. Wiesen and colleagues reported impaired ductal growth of EGFR-null mammary tissue transplanted from newborn mice into the suprarenal capsules of control mice (Sebastian *et al.*, 1998; Wiesen *et al.*, 1999). Since the penetrance and severity of defects in EGFR-null mice depend on the mouse strain, we analyzed mammary development from a strain that dies perinatally. Our study of EGFR-null embryonic mammary tissue demonstrated impaired ductal development in late fetal stages and thus extended the role of EGFR function to earlier stages. We have also confirmed that EGFR-null epithelium from a different strain background is able to grow and differentiate normally when introduced into wild-type stroma. Our experiments point to a necessary role for the GHR and EGFR signaling pathways specifically in the mesenchymal compartment of the mammary gland, while PrlR exerts its function specifically in the mammary epithelium. These results are consistent with our biochemical finding that GH and EGF can activate Stat5 in the cleared fat pad of a mammary gland. In contrast, Prl induced Stat5 phosphorylation in the epithelial but not in the stromal compartment of the mammary gland, supporting the prominent role of the PrlR in epithelial cell development.

While the function of most genes can be provided by one copy in the genome, both copies of the PrlR gene are needed to ensure the development of a functional gland. This haploinsufficiency is not the result of reduced gene expression due to imprinting but rather of the necessity of having the full complement of prolactin receptors (Ormandy *et al.*, 1997). Little mammary development is observed in the presence of 50% of the PrlR. In PrlR +/− mice in a mixed 129/C57BL/6 background functional mammary development occurs in the second pregnancy, demonstrating that either continuous hormonal stimulation can overcome the block or compensatory mechanisms are established. In contrast, no functional development occurs in the C57BL/6 background even after multiple pregnancies. In this genetic background alveolar development and Stat5 phosphorylation were impaired, and the expression of milk protein genes was reduced. In one case, limited lactation was observed after nine pregnancies, which correlated with appropriate Stat5 phosphorylation and milk protein gene expression. Based on our histological and biochemical data we suggest that development and differentiation of alveoli in PrlR-hemizygous mice are arrested during midpregnancy. Specifically, the alveolar cells do not activate the WAP gene at the level that is normally observed between days 14 and 16 of pregnancy. In contrast to the PrlR, no haploinsufficiency has been observed for Stat5a, Stat5b, Stat5a/5b, or Jak2 (our unpublished observations). This suggests that among the known signaling molecules in the prolactin pathway only the number of receptors is limiting. Since Stat5a, Stat5b, and Jak2 are shared with other cytokine signals, it is only prudent for the cell to provide an excess of these molecules.

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